Protein Kinase C-α Activity Inversely Modulates Invasion and Growth of Intestinal Cells*

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Eduard Batlle‡, Javier Verdúš, David Dominguez, Maria del Mont Llosas#, Víctor Díaz§, Noureddine Loukilii, Rosanna Paciucci, Francesc Alameda, and Antonio Garcia de Herreros**

From the Unitat de Biologia Cel.lular i Molecular, Institut Municipal d’Investigació Mèdica, Calle Dr. Aiguader 80, 08003 Barcelona, Spain

The phorbol ester phorbol 12-myristate 13-acetate induces remarkable phenotypic changes in intestinal HT-29 M6 cells; these changes consist of loss of homotypic adhesion and inactivation of E-cadherin. In parallel, cell growth is retarded. We have transfected HT-29 M6 cells with an activated form of the conventional protein kinase Cα (cPK-Cα). Expression of this isoform induced the acquisition of a scattered phenotype, similar to that adopted by cells after addition of phorbol 12-myristate 13-acetate, with very low cell-to-cell aggregation and undetectable levels of functional E-cadherin. These cell clones were highly motile and rapidly invaded embryonic chick heart fragments. Furthermore, cells expressing activated-cPK-Cα showed decreased proliferation in comparison to control clones. We have also studied how these two apparently antagonistic changes affect the tumorigenic ability of HT-29 M6 cells. When the different cell clones were xenografted into athymic mice, the effect on cell growth seemed to predominate. Expression of activated-cPK-Cα significantly reduced the size of the tumors; the cells with the highest level of expression did not even form subcutaneous tumors. Besides their smaller size, the morphology of these tumors was clearly different from those originated by HT-29 M6 cells, and they could be defined as infiltrative on anatomopathological basis. These results indicate that cPK-Cα controls both cell-to-cell adhesion and proliferation of intestinal cells.

Unveiling the mechanisms that control establishment and maintenance of intercellular contacts in the epithelium of the intestine is essential to understanding colon carcinogenesis. There is considerable evidence that protein kinase C (PK-C)1 isoforms are involved in this process; for example, addition of the phorbol ester phorbol 12-myristate 13-acetate (PMA) has been shown to reduce cell-to-cell contacts in epithelial cells (1).

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** To whom correspondence should be addressed. Tel: 3-221-1009; FAX: 3-221-3257; E-mail: agarcia@imim.es.

1 The abbreviations used are: PK-C, protein kinase C; Gf, PK-C inhibitor GF109203X; Go, PK-C inhibitor Go 6976; cPK-C, conventional PK-C; nPK-C, atypical PK-C; PMA, phorbol 12-myristate 13-acetate; uPA, urokinase-type plasminogen activator.

This compound has been widely used to activate PK-C in cells because it is membrane-permeable and causes a maintained stimulation of most members of this extended family (cPK-C and nPK-C) (2, 3). Addition of PMA to intestinal cell subpopulations derived from HT-29 cells, like its addition to many other epithelial cell line caused a significant stimulation of cell morphology and induces scattering of cell colonies (4). This process is characterized by the acquisition of a more fibroblastic phenotype, with lower cell-to-cell adhesion and inactivated E-cadherin (4, 5). In parallel, addition of this compound retards cell growth, an effect that has also been observed in other intestinal cells (6). Previous studies from our group have shown that cell scattering can also be induced by thymeleatoxin, a specific activator of conventional PK-Cs (cPK-Cs) (7). The goal of this study was to identify the PK-C isoform involved in these events and determine whether activation of this isoform in intestinal cells lead to altered tumorigenic properties of HT-29 M6 cells (HT-29 cell subpopulation isolated using 10^-6 methotrexate) in vivo.

EXPERIMENTAL PROCEDURES

Reagents—PMA, leupeptin and phenylmethylsulfonyl fluoride were supplied by Sigma. Gelatin and plasminogen were from Merck and Boehringer Mannheim, respectively. PK-C inhibitors GF109203X (Gf) and Go 6976 (Go) were purchased from Calbiochem (San Diego, CA); the other reagents were dissolved in dimethyl sulfoxide (Me2SO) and stored protected from light at -40 °C. [γ-32P]ATP was purchased from Amersham Pharmacia Biotech. Monoclonal antibodies against cPK-Cα and E-cadherin (HECD-1) were obtained from Transduction Laboratories (Lexington, KY) and Zymed Laboratories Inc. (San Francisco, CA), respectively. Prestained SDS-polyacrylamide gel electrophoresis molecular weight markers were from Bio-Rad (Richmond, CA). All of the oligonucleotides used in our assays were synthesized by Amersham Pharmacia Biotech.

Cell Culture—The properties of the cell line used in this study HT-29 M6 (M6), originally characterized with the name of HT-29 (10^-6 methotrexate), have been extensively described (8, 9). Cells were seeded at a density of 2 x 10^4 cells/cm^2 and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.) as described previously (5). Experiments of scattering were performed using cells 4–5 days after plating, when they were 40–50% confluent.

Mutagenesis of cPK-Cα and Transfection to M6 Cells—Human cPK-Cα was obtained from ATCC. Mutation of the alanine situated in position 25 to glutamic acid (A25E) was carried out according to the method of Kunkel et al. (10), using the oligo 5’-CCCGCAAAAGGGAGCCTAGCCAGAAGCG3’ (corresponding to nucleotides 89–114 of the sequence of human cPK-Cα). In addition to the change C to A (position 102, in bold), required to replace Ala with Glu, the G present in position 106 was mutated to a C (underlined); this second silent mutation generates a restriction site for SacI, which is useful to recognize the mutated cDNA. The presence of the mutations in the final construction was verified by sequencing using Sequenase (U. S. Biochemical Corp.). Mutated cPK-Cα, denominated cPK-Cα(+), was inserted in the NotI site of pOPRSV expression plasmid (Stratagene, La Jolla, CA) and transfected to M6 cells using LipofectAMINE (Life Technologies, Inc.) under the conditions indicated by the manufacturer. After 21 days of...
PKC-α regulates cell growth and invasion

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Inhibition of Gf and Go of the effects of PMA on M6 cells

<table>
<thead>
<tr>
<th>Additions to the cells</th>
<th>Scattering*</th>
<th>Homotypic adhesion*</th>
<th>Cytoskeleton-associated E-cadherin*</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>100</td>
<td>0</td>
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<tr>
<td>PMA</td>
<td>+ +</td>
<td>88 ± 12</td>
<td>96 ± 5</td>
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<tr>
<td>+ Gf (0.5 μM)</td>
<td>+</td>
<td>74 ± 10</td>
<td>92 ± 8</td>
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<tr>
<td>+ Gf (10 μM)</td>
<td>+</td>
<td>40 ± 12</td>
<td>110</td>
</tr>
<tr>
<td>PMA + Go (20 μM)</td>
<td>+ / –</td>
<td>90 ± 3</td>
<td>ND</td>
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<tr>
<td>+ Go (10 μM)</td>
<td>+</td>
<td>72 ± 13</td>
<td>100</td>
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<tr>
<td>+ Go (2.5 μM)</td>
<td>+</td>
<td>33 ± 15</td>
<td>10 ± 2</td>
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<tr>
<td>+ Go (0.6 μM)</td>
<td>+</td>
<td>7 ± 5</td>
<td>11 ± 3</td>
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* Cell scattering was examined under the phase contrast microscope by two independent researchers after 6 h of incubation; scattering was evaluated as + + (more than 90% of the colonies showed cells migrating out), + (20–90% of the colonies showed cell dispersion), + / – (cells flattened but did not move) or – (cells did not migrate and presented a phenotype very similar to untreated cells).

Homotypic adhesion (NTE/NTR index) was calculated by counting the number of cell particles after trypan blue staining in the presence (NTE) or absence (NTR) of Ca2+.

Cytoskeleton-associated E-cadherin was determined quantifying by Western blot and densitometry the presence of this protein in Triton X-100-insoluble fraction. The extent of downregulation of E-cadherin inactivation was blocked by similar concentrations of Go (0.2 μM) and inhibitors.

**RESULTS**

Inhibitors of cPK-C Isoforms block scattering of M6 cells induced by PMA—We have previously reported that PMA induces remarkable morphological changes in M6 cultures (4). After addition of this phorbol ester, M6 colonies scatter, and cells acquire a fibroblastic aspect. These effects are observed shortly after the addition of this phorbol ester at concentrations as low as 20 nM. Among the properties altered by PMA, cells lose homotypic adhesion, and concomitantly, E-cadherin is inactivated (5). These effects of PMA are prevented if the cells are simultaneously incubated with the PK-C inhibitor Gf (2 μM) (7). Gf is a bisindolylmaleimide derivative that selectively inhibits PK-C isoforms; in vitro, it shows a ranked order of potency of cPK-Cs > nPK-Cs > atypical PK-Cs (16). As shown in Table I, the action of PMA on cell scattering, homotypic adhesion, or E-cadherin inactivation was blocked by similar concentrations of Gf. The minimal dose of Gf required to prevent morphological scattering induced by PMA was 0.5 μM; in the presence of this concentration, cells incubated with PMA presented a similar phenotype to untreated controls (not shown). The effect on this inhibitor on the loss of homotypic adhesion was also estimated; IC50 was obtained with approximately 70 nM Gf, a concentration lower than the IC50 for nPK-Cs δ or ε activities (210 and 130 nM, respectively), determined by in vitro protein kinase assays. Concomitantly with the effect on homotypic aggregation, low doses of Gf (0.5 μM) PMA also blocks the decrease in E-cadherin-associated to cytoskeleton caused by PMA (Table I).
Recently, the preparation of a selective inhibitor of cPK-Cα with respect to nPK-C or atypical PK-C has been described (16). In vitro, this compound, Go, displays high activity against cPK-Cα and β (with IC₅₀ values of 2.5 and 6.2 nM, respectively) whereas no effect was observed on nPK-Cε or δ or atypical PK-C even at micromolar concentrations (16). In M6 cells, Go blocked the action of PMA on the three parameters studied (Table I), although the doses required were considerably higher than those of Gf. This difference could be explained by a lower ability to penetrate the cells; although the activity of Go and Gf have been studied in vitro, their relative potency in whole cells has not been characterized. However, even the highest concentration of Go used in our assays did not inhibit nPK-Cε activity in vitro (data not shown).

Expression of an Activated Form of cPK-Cα Causes Scattering of M6 Cells—The use of these inhibitors suggests that a cPK-C is responsible for triggering the process of scattering and loss of cell-to-cell adhesion. We have previously described that the only cPK-C expressed in HT-29 M6 cells is cPK-Cα (7). The presence of cPK-Cα, and not of cPK-Cβ or γ, was demonstrated both by Western blot with specific monoclonal antibodies and by reverse transcription-PCR analysis, using two oligos corresponding to consensus sequences of these three isoforms. Therefore, to prove the role of cPK-Cα in cell scattering, we have overexpressed this isoform in M6 cells.

A mutation in the pseudosubstrate domain of cPK-Cα was performed in order to obtain a higher basal activity of this enzyme in the absence of external activators. This mutation consists of the replacement of Ala (residue 25) present in the central position of the pseudosubstrate by a charged Glu residue. This substitution, A25E, activates cPK-Cα because it reduces the affinity of the autoinhibitory pseudosubstrate peptide for the catalytic site (17). Although considerably more active in the absence of effectors than the wild-type enzyme, this mutated cPK-Cα, denominated cPK-Cα(+), can be further activated by the addition of PMA and phospholipids (17). Four different cPK-Cα (+) transfectant clones were selected and analyzed in detail; these four clones summarized the different phenotypes obtained in our experiments. One of the clones (A1) presented a phenotype identical to control M6 (Fig. 1) and a similar sensitivity to PMA (not shown). Clone A2 showed a phenotype slightly less compact than control cells; the two other clones (A3 and A4) presented the phenotype previously defined as “scattered” to different extents. A3 cells formed colonies composed of flattened cells, with a low number of cell-to-cell contacts, whereas the phenotype of A4 cells was almost identical to that displayed by M6 chronically treated with PMA. In parallel, a transfection with a control plasmid that did not contain insert was performed; all of the clones observed presented a morphology identical to HT-29 M6 cells (not shown), and two of these clones (C1 and C2) were isolated and used as negative controls in our studies. Addition of the PK-C inhibitor Gf to clone A2 or A3 induced the formation of colonies identical to control cells; reversion by Gf, although important, was not complete in M6 cells chronically treated with PMA or in A4 cells (Fig. 1). Because the mutant cPK-Cα is not fully activated by the mutation and can be further stimulated by addition of PMA, we analyzed the sensitivity of the clones to the phorbol ester. Clones A2, A3, and A4 were sensitive to doses of PMA that did not induce scattering of any of the control clones; for instance, A2 cell colonies dispersed in response to 5–10 nM phorbol ester, a 4-fold lower dose than that required with control cells (Fig. 1, bottom row). In A4 and A3 cells, the addition of PMA induced the acquisition of an extremely stretched phenotype, characterized by long cellular extensions (Fig. 1, bottom row).

The expression of the mutant cPK-Cα was analyzed by RT-PCR; only clones A2, A3, and A4, but not A1 or the controls, showed expression of the mutant cPK-Cα (+) transcript (Fig. 2). Although the amplification was only semiquantitative, the rank of expression could be established as A4 > A3 > A2, demonstrating a correlation between expression of cPK-Cα (+) and cell scattering. Taking advantage of the new restriction site created with the mutation, the relative amounts of the wild-type and mutant forms were determined; only A4 presented levels of cPK-Cα (+) transcript higher than those of the wild-type form.

Because activation of cPK-Cα leads, in many cases, to down-regulation of this enzyme, determinations of total levels of this protein were not informative. However, the levels of other PMA-responsive PK-C isoforms were analyzed in transfectant clones by Western blot, to verify whether the higher sensitivity to PMA was due to increased synthesis of PK-Cα other than PK-Cα. We did not detect any increase in the levels or change in localization of the nPK-Cα ε and η in the cPK-Cα (+)-transflectant clones, nor was cPK-Cβ absent in M6, induced in these cells (data not shown).

In order to measure the endogenous activity of cPK-Cα, we employed an indirect assay: the analysis of expression of a gene containing phorbol ester-response elements. This method has been used previously by other authors with this goal (17, 18). The gene used in these studies was the urokinase-type plasminogen activator (uPA); the promoter of this gene contains several phorbol-ester responsive elements, and its expression is greatly stimulated by activation of several PK-C isoforms (19, 20). Therefore, in order to analyze endogenous cPK-Cα activity...
in the M6 cells and in the clones transfected with cPK-Ca(+) expression of uPA was determined by zymography. This method estimates the activity of uPA, a parameter that correlates with its expression. M6 or control clones (C1 and C2) presented very low levels of plasminogen activators. High levels of a protease activity with a molecular mass of 54 kDa, corresponding to uPA, were observed in the supernatant of these cells chronically treated with PMA (Fig. 3). A1 or A2 cells did not show significant levels of uPA; the presence of this activity was detected in A3 cells and, to a much greater extent, in A4 cells, where it was a level similar to that seen in M6 cells incubated with PMA (Fig. 3). These data allow us to rank the cells in terms of cPK-Ca activity as A4 > A3 > A2 = controls.

Expression of cPK-Ca(+) Modifies Other Cellular Properties Associated with Scattering: Transfectants Show Higher Mobility, Lower Cell-to-Cell Adhesion, and Decreased Levels of E-cadherin Compared to Controls—Scattering is associated with an increase in the mobility of cells. To determine whether expression of cPK-Ca enhanced mobility, a wound was inflicted in the epithelial monolayer, and the ability of the cells to fill the gap was determined. After 24 h, control M6 cells had not moved into the denuded area (Fig. 4). Incubation with 100 nM PMA but not with 10 nM PMA induced the cells to significantly fill the free space. Expression of cPK-Ca(+) correlated with the extent of the healing; A4 cells showed higher mobility than A3, and these in turn showed higher mobility than A2. A2 and A3 were sensitive to low doses of PMA (10 nM) that did not induce the movement of M6 cells (Fig. 4).

Besides the modification of other cellular properties, scattering requires the loss of homotypic aggregation. In M6 cells, the decrease in this parameter is concomitant with the inactivation of E-cadherin (5). Therefore, as expected, clones A3 and A4 showed dissociation indexes much higher than M6 cells or the control clones analyzed (A1, C1, and C2) (Table II). These indexes were very similar to that calculated for HT-29 M6 cells incubated in the presence of PMA. Clone A2 presented an index not significantly different from control cells, but these cells were much more sensitive to the addition of PMA (Table II). Very similar results were obtained when the function of E-cadherin was studied. Inactivation of this protein (loss of the association to the cytoskeleton) is reflected by an increase in its solubility in Triton X-100 (15). In untreated M6 cells and in the control clones, approximately 35% of total E-cadherin was insoluble in Triton X-100 (Fig. 5). However, in long-term PMA-treated cells or in A3 and A4 cells, the level of E-cadherin present in this fraction, which corresponds to the functional E-cadherin, was barely detectable (Fig. 5). The total levels of this protein were also lower, especially in M6 cells treated with the phorbol ester and in A4 cells (not shown).

Cell Growth Is Retarded by the Activation of cPK-Ca(+)—Another of the features that appear in M6 cells treated with PMA is a retardation in cell growth (4). As observed in Fig. 6, this effect of the phorbol ester requires concentrations significantly higher than those required to induce scattering. For instance, addition of 20 nM PMA, the lowest concentration that induced scattering, did not have any effect on the proliferation of HT-29 M6 cells. Growth of the different clones was measured either in the absence or in the presence of 20 and 100 nM PMA. Control cells (C1 and C2) grew with times of duplication similar to those of M6 cells and showed similar sensitivity to PMA. Minor differences were found between control and A2 cells either in the absence of PMA or in the presence of the two

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**Fig. 2. PCR analysis of the transfectants.** cDNA was obtained from the different clones and analyzed by PCR using oligos A1 and A2 (PCR A), B1 and B2 (PCR B), or actin-specific (Actin) as described under “Experimental Procedures.” A scheme of the construction with the position of the oligos is shown. The product of PCR A was transferred to a nylon membrane and analyzed with oligo B1 labeled with 32P; the product of PCR B was digested with SacI and analyzed with oligo A2. The figure shows representative results of PCRs A and B (autoradiograms) and of the amplification of the actin fragment used as control (ethidium bromide staining). The three amplifications were performed at low number of cycles (20–25 cycles) to avoid saturation of the reaction. In PCR B, a control of digestion of the plasmid with SacI was included; although it is not shown, the bottom fragment was not observed in M6 or A1 cells.

**Fig. 3. uPA activity in cPK-Ca(+) transfecants.** Media conditioned for 24 h from the different cell clones or M6 cells were treated when indicated with PMA because seeding were subjected to SDS-polyacrylamide gel electrophoresis in plasminogen and gelatin-containing gels, and protease activity was measured as described under “Experimental Procedures.” In addition of a protease of 55 kDa, corresponding to uPA, a minor band of lower molecular mass (probably consequence of the processing of uPA) was also detected in A4 cells.

**Fig. 4. cPK-Ca(+) expression increases mobility of M6 cells.** Cell mobility was determined using an assay of wound healing. Confluent cell clones were wounded with two cross-shaped scratches; PMA was added when indicated, and the ability of the cells to refill the gaps was examined after 24 h. The × 50 magnification micrographs show the results of one representative experiment of three performed.
different concentrations of this compound. As shown in Fig. 6, growth of A3 cells in absence of phorbol ester was similar to control. However, these cells were much more sensitive to the phorbol ester; 20 nM PMA inhibited proliferation of A3 by 50% versus less than 5% to M6 or C1 cells. The highest cPK-Cα(-) expressing clone, A4, proliferated at a lower rate and responded to low doses of PMA with a total block in proliferation (Fig. 6).

Expression of cPK-Cα(-) Stimulates Invasion of Chick Embryo Heart Fragments—The results obtained so far have indicated that activation of cPK-Cα induced the acquisition of a fibroblastic phenotype, with low functionality of E-cadherin and enhanced secretion of uPA, two alterations that have been related to a more invasive phenotype of epithelial cells (21, 22). Therefore, we analyzed whether transfection of cPK-Cα(-) had indeed enhanced the invasive properties of M6 cells, using the assay of invasion of embryonic chick heart fragments. Single-cell suspensions were added to the precultured heart fragments, and samples were processed after different periods of incubation. Histological analysis of cultures revealed striking differences between control cells and cells expressing cPK-Cα(-). M6 cells, or clone C1 cells, which behaved identically, gave rise to compact colonies that progressively occupied the peripheral parts of the heart fragment without invading the myocardial tissue (Fig. 7, G and H). Only after long times of confrontation (6 days) were small clusters of cells seen inside the heart tissue (not shown). In contrast, A4 cells were highly invasive; spindle-shaped A4 cells could be observed penetrating the heart tissue as early as 8 h after the initiation of the assay (Fig. 7, A and B). After 24 h, A4 cells had invaded the heart fragment and replaced extensive areas of this tissue (Fig. 7, C and D). These cells did not survive well in the co-culture; at longer periods of time (3–6 days), most of the A4 cells showed symptoms of cell damage, such as clumping of chromatin and shrinkage of cytoplasm (not shown). The invasive capability of A3 cells was also determined: these cells invaded more slowly than A4 cells, although an extensive colonization of the fragment was observed by 24 h (Fig. 7, E and F).

Ultrastructural sections of cultures revealed an active progression of A4 cells through the myocardial tissue, with a marked hypertrophy of their rough endoplasmatic reticulum and numerous cell extensions (Fig. 8, A and B). In contrast, M6 cells were localized in the periphery of the fragment and showed a polarized morphology with many mucous granules near the apical membrane and a microvilli (Fig. 8C). M6 cells lying in close apposition to the heart tissue showed a flattened morphology with no cytoplasmic processes.

Growth and Characteristics of Tumors Originated by M6 Cells Are Affected by Expression of cPK-Cα(-)—We have demonstrated that activation of cPK-Cα induces two alterations that are presumably antagonistic in the process of tumorigenesis: enhanced cell invasion and retarded proliferation. In order to analyze the tumorigenic ability of cells expressing cPK-Cα(-), the different clones and controls were xenografted into the subcutis of nude mice. Expression of cPK-Cα(-) was associated to a decrease in the size of the tumors originated by M6 cells (Fig. 9). In contrast to control C1 or A1 cells, which originated large tumors after eight weeks, A4 cells did not form macroscopic tumors at this late time (Table III). Absence of tumors was confirmed by microscopic analysis of the area of implantation after hematoxylin-eosin staining. No evidence of tumor cells was observed when the analysis was performed at longer (10 weeks) or shorter (10 days) times after grafting. Implantation of these cells in the spleen gave similar results (not shown).

The clones A2 and A3, with a lower expression of cPK-Cα(+), showed intermediate results. In both cases, xenografting gave rise to the formation of tumors, which were smaller than in the case of control cells. This decrease in tumor size correlated with the expression of cPK-Cα(-) (Table III).

The morphology of these A3-derived tumors, and in to a lesser extent A2 tumors, also showed differences with respect

![Fig. 5. cPK-Cα(+/-) transfectants present lower levels of E-cadherin associated to the cytoskeleton. Fractions soluble in Triton X-100 or only in SDS were prepared from different clones or M6 cells treated, when indicated, with PMA (100 nM) since seeding. Equivalent amounts of both fractions were analyzed by Western blot with an anti-E-cadherin monoclonal antibody; only a band of 120 kDa, corresponding to this protein, was detected. The figure shows a representative experiment of three performed.](http://www.jbc.org/)

![Fig. 6. Expression of cPK-Cα(-) retards cell growth. Proliferation of cells was measured during the exponential phase of growth directly by cell counting in the absence or in the presence of the indicated concentrations of PMA. The figure shows the mean of three different experiment, which did not differ by more than 5%. The number in parentheses corresponds to the duplication time of the cells in the different conditions studied.](http://www.jbc.org/)
to the controls. Control tumors presented well-defined limits without any sign of infiltration of neighboring tissues. These tumors grew expansively and pressed the skin without invading the dermis (Fig. 9, A and B). In some cases, probably due to their larger size, tumors showed areas of necrosis (Fig. 9A). In contrast, all of the A3 tumors examined were characterized by an irregular contour (Fig. 9, E–G). Areas in which the tumor cells have invaded the surrounding tissues were easily detected and comprised most of the periphery of the tumor. Single cells infiltrating the neighboring tissue (Fig. 9, E1 and G1) and muscle fibers surrounded by tumor cells (Fig. 9E2) were two characteristics of these A3 tumors. This infiltrative phenotype was also observed to a lesser extent in the A2 tumors but only in a small part of the periphery of 1 of the 16 controls analyzed. A noteworthy feature found in the A2 tumors was their ability to invade the dermis of the mice. As shown in Fig. 9, control tumors grew expansively without altering the skin structure (Fig. 9B1); A2 tumors infiltrate dermis and, in some cases, epidermis as well (Fig. 9, C, D, and D1). This invasion of the skin was not found in A3 tumors, probably due to their small size.

**DISCUSSION**

Several conclusions can be drawn from our results. First, it is evident that activation of cPK-Cα in M6 cells induces the acquisition of a fibroblastic phenotype, denominated scattered. Cell scattering is a consequence of co-ordinated changes that decrease cell-to-cell interactions, increase the adhesion to the extracellular matrix, and stimulate the migration of the cells. The basis of these phenotypic changes is alteration in the function of several proteins; for instance, inactivation of E-cadherin and increased uPA activity have been related to loss of homotypic adhesion and enhanced mobility, respectively (22, 23). We show here that expression of an activated form of cPK-Cα is sufficient to induce the acquisition of the scattered phenotype and to alterate the functionality of both E-cadherin and uPA.

Expression of cPK-Cα(+) also increased the ability of M6 cells to invade chick heart embryo fragments. Similar data have been obtained with other epithelial cells: overexpression of cPK-Cα in breast cells induced the acquisition of a fibroblastic phenotype and enhanced the proliferation rate and the tumorigenic and metastatic capabilities (24). However, differently to the results in breast cells, activation of cPK-Cα in intestinal M6 cells decreased proliferation and tumorigenicity.

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**Fig. 7.** Expression of cPK-Cα(+) stimulates invasion of chick heart fragments by M6 cells. The figure shows light micrographs of semithin sections from confrontations between precultured embryo heart fragments (marked H) and clones A4 (A–D), A3 (E and F), or M6 cells (G and H) fixed after 8 h (A and B), 24 h (C–F), or 3 days (G and H). T, M6 cells; arrowheads in A and B: A4 cells; asterisk in H: fibroblast showing signs of cell damage. Magnifications: A, C, E, and G: ×150; B, D, F, and H, × 600.

**Fig. 8.** Alterations in cell ultrastructure in cells expressing cPK-Cα(+) after invasion of EHF s. The figure shows representative transmission electron micrographs from some of the samples analyzed in Fig. 7. A, two A4 cells (T) actively progressing inside the heart tissue. H, myoblasts (×4500). B, detail from A showing an A4 cell (T) with a marked hypertrophy of rough endoplasmic reticulum cisternae (asterisk). Myofilaments (M) can be distinguished in neighboring myoblasts (H). Arrowheads, fuzzy material; arrow, Z-line (×15,000). C, M6 cells did not invade the heart tissue (H) after 3 days of culture. Arrow, mucosecretory granules; arrowheads, microvilli (×5000).
Similar data were obtained by Weinstein and co-workers (25) in HT-29 cells after transfection of another cPK-C, cPK-Cβ. Based on these data and the localization of this enzyme along the crypt-villus axis, a role for cPK-Cα in the negative control of cell growth in intestinal epithelium has been suggested (6, 26, 27). Association of cPK-Cα to the membrane has been detected by immunofluorescence in the mid-crypt region, where cells cease proliferation; this alteration has been suggested to reflect an increased activity of this enzyme, although this conclusion has not been definitively proved.

It is remarkable that activation of the same enzyme, cPK-Cα, exerts two different actions on M6 cells: it increases invasion and retards cell growth, two effects that seem antagonistic in tumor development. The results presented in this report demonstrate that although both effects are triggered by activation of the same PK-C isoform, they are differentially sensitive to the extent of this activation. A moderate stimulation of the enzyme (for instance, present in A3 cells) causes an increase in the ability of these cells to infiltrate surrounding tissues and a decrease in the growth of the tumor; on the other hand, when the activation of the enzyme is extensive (A4 cells), the growth inhibitory effect is predominant, and the tumor is unable to develop. These effects mimic what happens in M6 cells treated with PMA: cell scattering and loss of cell-to-cell contacts require low doses of this phorbol ester, whereas higher concentrations are required to affect cell growth. Similar results were obtained with other intestinal cell lines with respect to the sensitivity of these two parameters to PMA. In all of these cell lines (WiDr, Caco-2, HRT18, SW620, and SWCo15), maximal inhibition of growth (26–52%, depending on the cell lines) required concentrations of the phorbol ester about 100 nm, whereas scattering was observed at much lower doses.2

Several lines of evidence indicate that alterations in PK-C may be involved in malignant transformation of colon in humans. Colon adenocarcinomas show a reduction in PK-C activity compared with normal adjacent mucosa, indicating down-regulation of this enzyme in the tumors (28–30). Experimental models of colon carcinogenesis have provided evidence of changes in PK-C both in premalignant and malignant epithelial cells; these changes include translocation of PK-C and subsequent down-regulation of this enzyme (31). Alterations in the content of specific PK-C isozymes have been also described in colon tumors with respect to normal tissue either in human samples or in experimental animal models (32, 33); however, different laboratories have reported contrary results of these analyses (32, 34, 35). Therefore, at the present, it is not clear whether the initial activation or the later down-regulation are related to colon tumorigenesis, neither the role of specific isoforms in this process.

The results presented here can help to explain the role of cPK-Cα in colon carcinogenesis. This enzyme might exert a dual action: 1) a moderate activation of cPK-Cα would take place at the first stages of carcinogenesis, being involved in the loss of function of E-cadherin, and 2) a later down-regulation and inactivation of cPK-Cα would result in an uncontrolled growth of the primary tumor or the metastasis. According to this model, studies performed with cell lines or tumors that have progressed beyond the first stage would not show any positive effect of PK-Cα activation in tumor development. Experiments directed to the validation of this model using transgenic animals are currently in progress in our laboratory.

While this work was being written, the article by Rosson et al. (36) came to our notice; it describes the involvement of

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2 E. Batlle and A. Garcia de Herreros, unpublished observations.
cPK-Cα in the scattering of LLC-PK1 cells induced by PMA. Their conclusion was obtained by expression of the wild-type form and a negative mutant of cPK-Cα in these cells. Although overexpression of a dominant negative mutant does not demonstrate involvement of cPK-Cα in cell scattering induced by PMA, the results presented by these authors are totally consistent with those described in this work.

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Protein Kinase C-α Activity Inversely Modulates Invasion and Growth of Intestinal Cells

Eduard Batlle, Javier Verdú, David Domínguez, Maria del Mont Llosas, Víctor Diáz, Noureddine Loukili, Rosanna Paciucci, Francesc Alameda and Antonio García de Herreros

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